

A twist in anti-inflammation: Annexin 1 acts via the lipoxin A₄ receptor[☆]

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Abstract

The inflammatory response is a life-saving protective process mounted by the body to overcome pathogen infection and injury; however, in chronic inflammatory pathologies this response can become deregulated. The existence of specialized anti-inflammatory pathways/mediators that operate in the body to down-regulate inflammation have now emerged. Thus, persistence of inflammation leading to pathology could be due to malfunctioning of one or more of these counter-regulatory pathways. Here we focus on one of them, the anti-inflammatory mediator annexin 1, and provide an update on its inhibitory effects upon the leukocyte trafficking process. In particular, recent evidence that receptors of the formyl-peptide family, which includes also the lipoxin A₄ receptor, could be the annexin 1 receptor(s) in the context of anti-inflammation might provide new avenues for exploiting this pathway for drug discovery.

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1. The attraction of anti-inflammation

The initial events characteristic of the host inflammatory response occur in the microcirculation and consist of alteration in permeability of the vessel wall and trafficking of blood cells to surrounding tissues. The latter process is of major importance in terms of final efficacy against the inflammatory insult and with respect to the transition from innate immunity to adaptive immunity [1]. The early steps of the white blood cell recruitment process have been partially solved demonstrating the coordinated action of classes of adhesion molecules and leukocyte activators [2]. However, it is crucial to host survival that not only the inflammatory reaction to exogenous insults is mounted rapidly and

efficiently, but also that it subsides in a temporal fashion.

Research over the past years has revealed the existence of several checkpoints set up by the host to assure adherence to a strict time-profile of the inflammatory response. A few years ago we reviewed some of those operating with the microenvironment of an extravasating leukocyte in contact with the activated endothelium of the post-capillary venule [3]. It is plausible that several extracellular and intracellular mechanisms are disseminated within and outside the vasculature and are actively involved in restoring homeostasis; for instance, with respect to a resident cell like the macrophage, it is emerged that it may change its phenotype towards an *anti-inflammatory* mode [4] a phenotype also associated with active removal of apoptotic leukocytes [5].

Thus, the concept of anti-inflammation has been put forward and it covers the elucidation of mechanisms that operate in the host during the resolution phase of the inflammatory response, thereby assuring its self-limiting nature [6]. This *holistic approach* is based on the concept that disturbances in one or more of these

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counter-regulatory circuits could lead to exacerbated inflammatory responses just as effectively as over-activation of the pro-inflammatory mediators, e.g. cytokines and adhesion molecules. Thus, from a drug discovery point of view, development of agonists to mimic the effects of a specific anti-inflammatory/pro-resolving mediator could be as effective as trying to block the actions of a given pro-inflammatory substance. It is envisaged that drugs depicted on a pathway that operates in the host to switch off inflammation are likely to have better compliance producing less side effects.

Some of these anti-inflammatory mechanisms have been studied in models of acute resolving innate immune response [3,7–9] demonstrating a role for mediators such as cortisol (or corticosterone in rodents), adenosine, tumour necrosis factor stimulated gene 6, lipoxin A₄ (LXA₄) and its more stable epimers produced by aspirin-inhibition of cyclo-oxygenase-2 (COX-2), heme oxygenase, low concentrations of nitric oxide, galectin-1, and interleukin-10 to name just a few [10]. Within this group of substances, the glucocorticoid-regulated protein annexin 1 (Anx-A1) has carved itself an important role.

2. Annexin 1

2.1. Annexin 1: An endogenous anti-inflammatory mediator

The observation that glucocorticoids could inhibit prostaglandin release with a mechanism different from aspirin (i.e. overcome by addition of the COX substrate, arachidonic acid) led to the discovery of a 37 kDa protein, termed macrocortin, renocortin or lipomodulin, before being christened lipocortin 1 when cloned in 1986 [11]. Application of human recombinant lipocortin 1 (subsequently renamed annexin 1, the first member of a superfamily of structurally related proteins) in models of acute and chronic inflammation replicated some of the anti-inflammatory actions of glucocorticoids [12].

Endogenous annexin 1 plays a tonic inhibitory role on the host inflammatory reaction, such that a prolongation of this response is observed in animals deficient of the annexin 1 gene or passively immunized with neutralising antibodies to the protein [13,14]. In an in vitro context, several cell types have been shown to produce annexin 1 and reported to respond to pharmacological doses of the protein or its bioactive peptides (see below), including macrophages and microglia cells, neutrophils and epithelial cells to name just a few [10,15–17]. Of interest here, out of a protein more than 300 amino acid long, small peptides drawn for the N-terminal region retain biological activity, and since the annexin 1 N-terminus is not homologous to other

members of the family [18], it is likely that these peptides reproduce solely annexin 1's biological effects. As discussed further, peptides termed Ac2-26, Ac2-12 (Fig. 1) or Ac9-25 (Fig. 1) have been reported to retain most of the effects of the full-length protein in in vivo and in vitro systems.

Annexin 1 expression is particularly abundant in cells pivotal to innate immunity, including blood neutrophils and monocytes [19,20], macrophages and epithelial cells [21]. Some time ago, we unravelled an important mechanism for annexin 1 secretion (with the exception of the prostate gland [22], many doubted the protein could be released from cells since it was lacking a canonical signal peptide), showing that human neutrophil adhesion to endothelial monolayers in vitro mobilized large amounts of the protein on the leukocyte cell surface [23]. Annexin 1 externalization on the cell surface of adherent neutrophils, explained by a marked localization of the protein in the matrix of gelatinase granules [24,25], provided a rationale for the efficacy of anti-annexin 1 blocking antibodies in abrogating dexamethasone-induced delay in leukocyte emigration [26]. In addition, the entire “annexin 1 system” seemed activated in the context of neutrophil adhesion to the endothelium, since evidence for a catabolic process was obtained, leading us to postulate the existence of a specific *lipocortinase* [3].

To conclude this section, we then postulated that once on the cell surface, neutrophil-derived annexin 1 might act in an unclear autocrine/paracrine manner to inhibit the process of leukocyte extravasation [23], but the mechanism was not clear!

2.2. Annexin 1 acts at the lipoxin A₄ receptor

Using specific rabbit sera, neutrophil-derived annexin 1 could be immunoprecipitated with the LXA₄ receptor (ALXR) selectively when the leukocytes were adhered to endothelial monolayers [27]. The physical interaction, and its specificity, between these two proteins was strong evidence for a shared anti-inflammatory target, this seven trans-membrane domain G protein coupled receptor, between two distinct endogenous anti-inflammatory mediators. The biochemical interaction between annexin 1 and ALXR was supported by experiments of binding using transfected cells or primary neutrophils. In addition, heterologous displacements in the binding assays were observed when using LXA₄ or the annexin 1 mimetic, the bio-active peptide Ac2-26 [28], as tracers [27].

Somehow puzzling, ALXR belongs to a family of human receptors that includes also the formyl-peptide receptor (FPR) and another receptor analogue FPR-like 2 (FPRL-2) [29]. Peptides derived from N-terminus of annexin 1 have also been reported to activate FPR as determined by receptor internalization in transfected

cells and by blocking its actions with putative FPR antagonists [30]. More recently, the ability of peptide Ac2-26 to interact with and activate all three members of the human FPR family has been reported. In this study, peptide Ac2-26 promoted calcium fluxes and chemotaxis of human kidney embryo cells transfected with each single receptor [31]. Two considerations are now worthwhile. First, it is unclear if full-length annexin 1 shares with its N-terminal peptides the ability to activate FPR and FPRL-2, whereas it clearly binds to ALXR and competes with LXA₄ binding. It is possible that the short 24 or 15 amino acid long peptides might assume several conformations otherwise restricted once the sequence is part of the larger structure (the full protein is 346 amino acid long). The fact that the list of ligands binding to FPR and FPRL-2 is ever growing does favour this possibility. Second, the FPR antagonists mostly used in these studies, the so-called Boc (butoxyl-carbonyl) compounds, do not really discriminate between FPR and ALXR (and possibly FPRL-2); for instance, a butoxyl-carbonyl derivative blocked the effect of serum amyloid protein A on ALXR [27]. Thus, the ability of these antagonists to block specific actions of the full-length protein on human neutrophils, e.g. calcium fluxes and L-selectin shedding, does not indicate selective FPR involvement, and conversely does not exclude ALXR-mediated effects [32]. Similar partial conclusions could be reached with respect to endogen-

ous annexin 1 effects upon removal of apoptotic neutrophils by human macrophages [33].

In the context of the human receptor, it is worth recalling that LXA₄ and its analogues have opposite effects to annexin 1 on human peripheral blood adhesion molecule expression. So, whereas annexin 1 [32], and its N-terminal peptides [30], cause shedding of L-selectin following addition to neutrophils (and monocytes) in vitro, LXA₄ and its analogues augment basal cell surface levels of L-selectin [34], indicating possibly the existence of a constitutive cleavage of this lectin binding protein. Peptide Ac2-26 and LXA₄ displayed opposite effects also in an artificial system in which ALXR was coupled to Gq to promote cell chemotaxis [27]. Recent advances in receptor pharmacology are accommodating these apparent discrepancies, teaching us how the same receptor might assume ligand-specific conformation thereby transducing ligand-specific signal transduction responses that yield specific cellular effects [35]. Table 1 illustrates a non-exhaustive list of ligands for human FPR, ALXR and FPRL-2, including the recently reported heme-binding protein fragment for the latter receptor type [36].

As a final note for this section, in our working model, the hypothesis that externalized annexin 1 would act in an autocrine/paracrine manner on the adherent leukocyte has often been put forward [10]; however, endothelial cells have also been shown to express ALXR

Table 1
Non-exhaustive pattern of human N-formylpeptide receptors ligands and cellular expression

Receptor	Agonists	Expression
FPR	<ul style="list-style-type: none"> ● fMLP (nM concentration) ● WKYMVm (W peptide) ● HIV gp41-derived peptides ● Deoxycholic acid ● Chenodeoxycholic acid 	Neutrophils–monocytes–dendritic cells Hepatocytes–Kupffer cells Epithelial cells–astrocytes Smooth muscle cells–endothelial cells Neuroblastoma
ALXR	<ul style="list-style-type: none"> ● Lipoxin A₄ ● Serum Amyloid A ● fMLP (μM concentration) ● Beta amyloid fragment ● Humanin ● Formylated Humanin ● PrP 106-126 peptide ● Urokinase receptor fragment ● Annexin 1 ● HIV peptides ● <i>H.pilori</i> peptide ● WKYMVm (W peptide) 	Neutrophils–monocytes Immature dendritic cells–T cell Epithelial cells–endothelial cells Synovial fibroblasts Microglia–astrocytes–
FPRL-2	<ul style="list-style-type: none"> ● F2L (derived from an heme-binding protein) ● WKYMVm (W peptide) ● Humanin ● <i>H.pilori</i> peptide 	Macrophages–monocytes Dendritic cells

Table extracted as a rapid summary from the following Refs. [36,52,63–67].

[37], along with specific binding sites for annexin 1 [38], and have been reported to re-uptake annexin 1, possibly in its cleaved isoform, from emigrating leukocytes [25].

2.3. Annexin 1 receptor in experimental inflammation

Use of the technique of intravital microscopy has helped us to demonstrate the effect of pharmacological doses of annexin 1 and its bioactive peptides on specific steps of the leukocyte extravasation process. Administration of either ligand caused no effect on the extent of leukocyte rolling in the mouse mesentery, but instead it caused an alteration of the fate of adherent cells [39,40]. In analogy with what was originally reported for dexamethasone in the hamster cheek pouch microcirculation [26], intravenous treatment with annexin 1 or peptide Ac2-26 provoked detachment of previously adherent cells, with a rapid kinetics (~2–10 min) [39,40]. It is yet unclear whether shedding of L-selectin, demonstrated with human cells in vitro, could be of any relevance in these experimental conditions.

These data produced by intravital microscopy support the tissue-protective effects displayed by annexin 1

and peptide Ac2-26, as seen following an ischemia-reperfusion procedure in the mesenteric vascular bed of the rat [41], in models of heart infarct both in rats [42,43] and mice [44] as well as following focal ischemia of the brain microcirculation (Gavins FNE et al., unpublished data). Interestingly, LXA₄ analogues are effective in controlling post-reperfusion tissue injury and they also prevent secondary organ injury [45]. In the mouse, an epi-lipoxin produces detachment of adherent leukocytes with a kinetics similar to that produced by peptide Ac2-26 [39,40]. But, which receptor(s) is responsible for these protective, anti-adhesive effects of annexin 1?

The family of murine FPR is even more complex than its human counterpart when one considers that there are currently eight related genes: murine (m)*Fpr*, *Fpr-rs1*, *Fpr-rs2*, *Fpr-rs3*, *Fpr-rs4*, *Fpr-rs5*, *Fpr-rs6* and *Fpr-rs7*. Gao et al. [46] proposed that *mFpr* is the orthologue of human FPR. In contrast, the human ALXR (sometimes referred to also as FPRL-1) gene splits into *Fpr-rs1*, which seems to be identical to mouse receptor identified for LXA₄, [47] and *Fpr-rs2*, which mediates the actions of serum amyloid A [48]. It is unclear if all other genes yield receptor protein, though the list has recently been

Table 2
Non-exhaustive pattern of mouse N-formylpeptide receptors ligands and cellular/tissue expression

Receptor type	Agonists	Cellular and tissue expression (mRNA or protein)
Fpr1	<ul style="list-style-type: none"> • fMLP (low μM affinity) • Peptide Ac2-26 • WKYMVm (W peptide) 	Neutrophils–monocytes–spleen–lung–liver
ALXR (Fpr-rs1)	<ul style="list-style-type: none"> • fMLP (high μM affinity) • Lipoxin A₄ • Annexin 1 	Neutrophils–monocytes Microglia Spleen–lung Heart–liver
Alternate ALXR (8C10)	<ul style="list-style-type: none"> • Lipoxin A₄ 	Lung–spleen–adipose tissue
Fpr-rs2	<ul style="list-style-type: none"> • Serum Amyloid Protein A • HIV peptides • WKYMVm (W peptide) • fMLP (high μM affinity) 	Neutrophils–microglia Spleen–lung
Fpr-rs3	<ul style="list-style-type: none"> • (None to date) 	Brain Skeletal muscle
Fpr-rs4	<ul style="list-style-type: none"> • (None to date) 	
Fpr-rs5	<ul style="list-style-type: none"> • (None to date) 	
Fpr-rs6	<ul style="list-style-type: none"> • (None to date) 	Brain–spleen–testis Skeletal muscle
Fpr-rs7	<ul style="list-style-type: none"> • (None to date) 	Heart–liver–lung

Table extracted as a rapid summary from the following Refs. [40,44,46–49,68].

augmented by the identification of a receptor homologue to mouse ALXR, functionally activated by LXA₄ and partially modulated by experimental endotoxemia [49]. These findings clearly indicate potential problems in homogeneity of the nomenclature, as well as in identifying potential tissue specific receptors responsible for the protective effects of annexin 1 and its mimetics. In terms of tools available, we have used putative FPR receptor antagonists and mice knocked out for *mFPR* [50] to address these aspects. Table 2 summarizes the murine family of FPR in a succinct manner.

2.4. Studies with genetically modified mice

Mouse *FPR* displays a significantly lower affinity for its putative agonist formyl-Met-Leu-Phe (fMLP), since activated at micromolar concentrations [51] versus the nanomolar concentrations required for the human receptor [52]. Using blood taken from wild type and *FPR* null mice, and measuring CD11b up-regulation in response to formyl-peptides and platelet-activating factor, we could determine that: (i) peptide Ac2-26 (up to ~60 μM) did not activate neutrophils taken from either genotype; (ii) peptide Ac2-26 and Boc derivatives blocked the effects of formyl-peptide both on wild type and *FPR* null neutrophils. In analogy with what reported for purified neutrophils and cells transfected with *mFPR* or *FPR-rs1* [51], fMLP activated the former receptor at 3 μM concentration, and the latter at ~30 μM concentration [40]. In addition, the annexin 1 peptide displayed a very good degree of selectivity, mildly affecting CD11b up-regulation produced by a lipid cell activator.

The combined ability of fMLP and Boc-derivatives to produce cellular responses in cells and mice lacking *FPR* provided molecular explanation for other studies. Using the model of zymosan peritonitis, peptide Ac2-26 lost most of its anti-migratory effect in *FPR* null mice, whereas the anti-migratory action of the full length annexin 1 was only partially altered [53]. In either case, though, Boc derivatives blocked the anti-inflammatory response. These data are now clearer since the involvement of more than one receptor of the family can be advocated. In line with the latter conclusion, a study of intravital microscopy of the mouse mesenteric microcirculation revealed that peptide Ac2-26 inhibition of cell adhesion, as assessed at a fixed time-point (45 min post-reperfusion), was only partially removed by gene deletion of *mFPR*. In contrast, the detachment effect of peptide Ac2-26 (discussed above) was intact in *FPR* null mice [40]. Finally, when the leukocyte trafficking across the post-capillary venule endothelium was assessed in the absence of peptide Ac2-26 administration, thus by simple comparison of the quantitative response in wild type and *FPR* null mice, a higher degree of cell emigration, as measured in the sub-endothelial matrix,

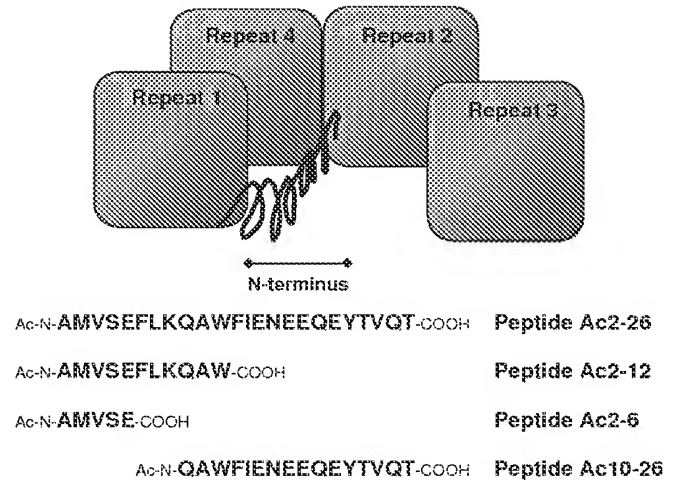


Fig. 1. Schematic representation of annexin 1 and its N-terminal derived bioactive peptides. The four repeat structure of annexin 1 is shown with the pairing of repeat 1 and 4, and repeat 2 with repeat 3. Each repeat consists of 70–80 amino acids. The N-terminal domain, attached to repeat 1, emerges from the concave face of the “donut structure” of annexin 1 (as defined by X-ray crystallization [70]). Annexin 1 binding to calcium cations, and likely also to phospholipids, causes a re-arrangement with consequent exposure of the N-terminal alpha helix [71], making this region more available for potential interaction with protein counterparts (e.g. receptors). Below the annexin 1 schematic picture, sequences of bioactive N-terminal-derived peptides shown to interact with members of the FPR family of receptors (see Refs. [27,30,31,40,53]), are illustrated.

was measured in the latter group of mice. It therefore seems that, in the mouse mesentery, both *FPR* and ALXR are present and may produce distinct effects on specific events occurring during the initial stages of the leukocyte-endothelium interaction. Recent work in the mouse cremaster microcirculation inflamed with platelet activating factor indicates a lack of involvement of *FPR*: peptide Ac2-26 produced a marked inhibition of cell emigration (but not cell adhesion), and this effect was fully retained in *FPR* null mice (Fig. 2). Also, the latter genotype did not display any significant response when compared to wild type mice (Fig. 2). Thus, studying receptor mechanisms in distinct district is of crucial importance.

Much work has been performed to dissect the early events of post myocardial infarct. Ischemia reperfusion of the heart is one of the most important underlying causes of cardiovascular diseases such as myocardial infarction, thrombotic stroke, embolic vascular occlusion and peripheral vascular injury. The infiltration of blood-born polymorphonuclear leukocytes [54–56] is possibly linked to the tissue damage associated with reperfusion injury as well as other vascular injuries, although this remains controversial [56,57]. Genetically altered mice have long been used as important tools to study leukocyte adhesion in cardiovascular inflammation [58]. At variance from the mesenteric microcirculation, testing the effect of peptide Ac2-26 in the mouse

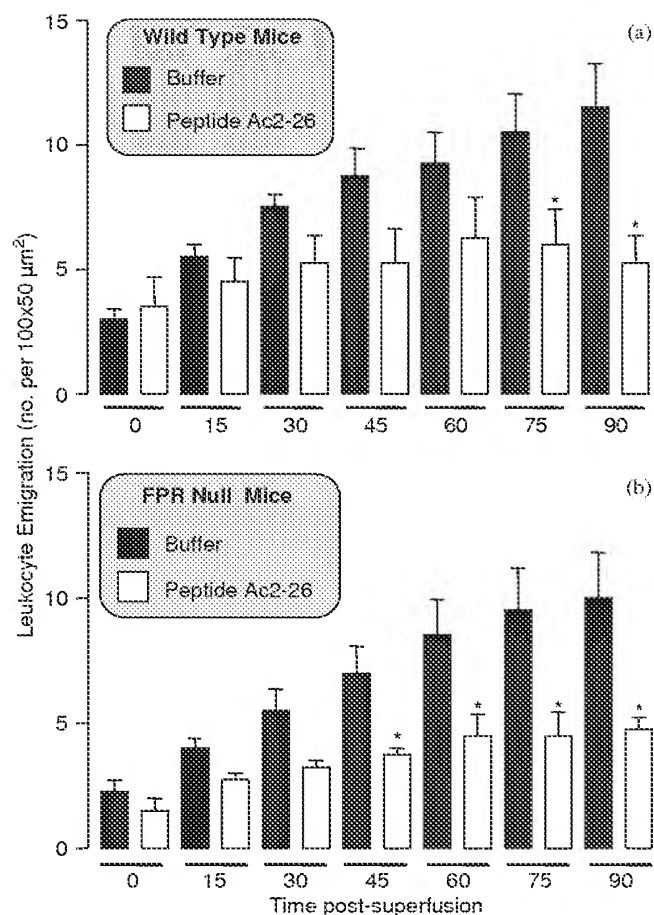


Fig. 2. Effect of peptide Ac2-26 on leukocyte emigration in the mouse cremaster microcirculation. Mouse cremaster microvascular beds were inflamed by continuous superfusion with 100 nM platelet-activating factor (starting at time 0) and the extent of cell emigration quantified at the reported time-points. (a) Cell emigration as determined in C57BL/6 mice after treatment with buffer (100 μl i.v.) or peptide Ac2-26 (100 μg i.v. at time 0). (b) As in panel A, but using FPR null mice. Data are mean \pm SEM of 6 mice per group. * $P < 0.05$ vs. respective buffer group.

myocardial infarct model revealed unexpected differences. In analogy to what was seen in the rat model [43], peptide Ac2-26 produced a cardio-protective effect in C57BL/6 mice, an effect evident at comparable doses to those required in the rat (1 mg/kg or $\sim 30 \mu\text{g}$ per mouse; considering $\sim 6 \text{ ml}$ of total blood volume, this corresponds to $5 \mu\text{g/ml}$ or an initial concentration of $1.5 \mu\text{M}$). It is important to note that the full-length protein is highly potent in inhibiting heart damage, and the N-terminal region is crucial to this effect, as seen with chimaeric proteins [42]. Also, in this acute experimental condition, administration of a Boc derivative alone was ineffective, but it blocked the cardio-protection afforded by peptide Ac2-26 [44]. Identical results were obtained with FPR null mice: they displayed a similar degree of acute heart damage (thus excluding a tonic inhibitory role for this receptor); a full cardio-protective of peptide

Ac2-26 was attained (not dissimilar from that measured in wild type mice), and this protection was lost by co-administration of a Boc compound.

Heart [44] and mesenteric tissue [40] samples express ALXR mRNA and protein, both in basal condition and after ischemia/reperfusion: the semi-quantitative pattern of expression seem similar between wild type and FPR null mice indicating that compensatory mechanisms are unlikely to have occurred in these mice following targeted gene disruption. The actual role played by mouse ALXR in the anti-inflammatory effects of LXA_4 has been revealed in many different models of inflammation, from acute air pouches [59] to a model of nephritis [60,61]. In the mouse myocardial infarct model, administration of a LXA_4 analogue or of peptide W (active at mouse ALXR) afforded protection in FPR null mice, to a similar degree as peptide Ac2-26 [44]. The current status of the role of annexin 1 receptor(s) in experimental model of pathology is summarized in Table 3.

Recent in-vitro and ex-vivo evidence showed that peptide Ac2-26 can have direct protective actions in isolated heart papillary muscles and cardiomyocytes [62]. Clearly there are no neutrophils in this condition, though, based on RT-PCR analysis of untreated hearts [44] ALXR might be expressed. Finally, it is worth recalling that human recombinant annexin 1 did not exert cardio-protection in the rat isolated Langerdoff preparation [42].

3. Conclusive remarks

Much work has been performed in the last 20 years concerning the biology of annexin 1. This endogenous anti-inflammatory mediator produces an important inhibitory checkpoint on the process of leukocyte extravasation and the tissue injury that ensues, with the main active region, or pharmacophore, residing in its N-terminus. Over the years we have developed a model in which the circulating leukocyte (mainly neutrophils) is not only the source but also the target for annexin 1 and its bioactive peptides (Fig. 1), with ALXR being the receptor mostly responsible for these anti-inflammatory actions. However, like a great deal of instances in science, the story is a great deal more complex. Although it seems accepted that ALXR mediates several anti-inflammatory actions of annexin 1 and N-terminal-derived peptides in experimental model, the complexity of the murine FPR family and the paucity of tools available (e.g. genetically deficient mice) favour a degree of caution. Much more work needs to be done, and one picture that is slowly emerging indicates tissue/cell specificity for the receptor sub-type mediating the major effects of these anti-inflammatory agents.

Table 3

Annexin 1 and FPR functional interaction as determined in in-vivo and in-vitro experimental systems^a

Model	Species	System	Effect of Anx-A1 or peptide(s)	Boc-sensitive	Involvement of		Refs
					FPR	ALXR	
Zymosan							
Peritonitis	Mouse	In-vivo	↓ Cell migration	Yes	50 ^b	NT	[53]
Heart IR	Rat	In-vivo	↓ Tissue damage	Yes	NT	NT	[43]
Heart IR	Mouse	In-vivo	↓ Tissue damage	Yes	No	Likely ^c	[44]
Mesentery IR	Mouse	In-vivo	↓ Cell adhesion	Yes	50 ^b	Likely ^c	[40]
Mesentery IR	Mouse	In-vivo	↑ Cell detachment	Yes	No	Yes	[40]
Mesentery IR	Mouse	In-vivo	↓ Plasma leakage	Yes	No	NT	[40]
Pituitary Gland	Mouse	Ex-vivo	↓ Peptide release	Yes	No	NT	[69]
Neutrophil	Human	In-vitro	↑ Ca ²⁺ fluxes	Yes	Yes	Yes	[30,31]
Neutrophil	Human	In-vitro	↑ CD62L shedding	Yes	NT	NT	[30,32]
Neutrophil	Human	In-vitro	↓ Chemotaxis	Yes	NT	NT	[30]

^aAbbreviations: ALXR, lipoxin A₄ receptor; Boc derivative, butoxy-carbonyl derivative of formyl-Met-Leu-Phe; FPR, formyl peptide receptor; IR, ischaemia reperfusion; NT, not tested.

^bPercentage reduction in FPR-null mice.

^cThe efficacy of Boc derivative indicates that the non-FPR receptor involved might be ALXR.

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